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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

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TITLE OF THE INVENTION (500 characters max)

MicroRNA Molecules Specifically Expressed in Human Embryonic Stem Cells

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MicroRNA molecules specifically expressed in human embryonic stem cells

Field of the Invention

The present invention relates to novel MicroRNA molecules specifically expressed in human embryonic stem cells.

Background of the Invention

Embryonic stem (ES) cell lines were first derived from mice and are now available from a variety of mammalian systems, including human. They are characterized by nearly unlimited self-renewal in an undifferentiated state under defined culture conditions while retaining differentiation capacity (Evans and Kaufman, 1981; Martin, 1981; Smith, 2001). During differentiation in vitro, ES cells are able to develop into various kinds of specialized somatic cell types and recapitulate processes of early embryonic development. Thus, ES cells hold promise as an unlimited source for various clinical and biotechnological applications (Brustle, 1999; Li et al., 1998; Martin, 1981; Pera et al., 2000).

ES cell lines were established first in mice in 1981 (Evans and Kaufman, 1981; Martin,1981) and have been used as a model system to study mammalian ES cells. Currently a few molecular regulators are known to participate in the self-renewal and pluripotency of mouse ES (mES) cells. A POU family transcription

factor Oct4, the classical marker of all pluripotent cells, is specifically expressed in preimplantation embryos, epiblast, germ cells and pluripotent stem cell lines including ES cells, embryonic germ (EG) cells and embryonic carcinoma (EC) cells (Palmieri et al., 1994; Yeom et al., 1996). Oct4 plays a critical role in the establishment and maintenance of pluripotent cells in a pluripotent state (Nichols et al., 1998; Niwa et al. 2000; Pesce et al., 1998). Leukemia inhibitory factor (LIF) can maintain self-renewal of mES cells through activation of Stat3 (Niwa et al., 1998). Oct4 and Stat3 each interact with various cofactors and regulate the expression of multiple target genes (Niwa, 2001). Two other transcription factors, Sox2 and FoxD3, have been shown to be essential for pluripotency in mice embryos (Avilion et al., 2003; Hanna et al., 2002). More recently, it was found that the homeoprotein Nanog is capable of maintaining mES cell self-renewal independently of LIF/Stat3 (Chambers et al., 2003; Mitsui et al., 2003).

The first human ES (hES) cell line was established only recently (Thomson et al., 1998) and 12 lines are publicly available worldwide (NIH Human Embryonic Stem Cell Registry). Despite their great potential, hES cells have not been a prolific source of information. This is mainly due to the technical difficulties in cell culture. Maintaining and expanding hES cells requires laborious and skill-intensive procedures. Moreover, the population-doubling time of hES cells is almost three times longer than that of mES cells (Amit et al., 2000). There exist apparent differences in the characteristics of hES cells compared to mES cells in many aspects, including the regulation of self-renewal. Of the regulators found in mice, only a few including Oct4 play similar regulatory roles in hES cells. Others

such as LIF do not affect hES cells in maintaining their self-renewal (Reubinoff et al., 2000). Dissecting the regulatory mechanism in hES cells will greatly enhance the understanding of stem cells as well as their application.

Recent advances in small RNA research have implicated microRNAs (miRNAs) as important regulators of development. miRNAs constitute a large family of non-coding small RNAs of ~22 nucleotides (nt) in length. Our understanding of miRNA function originates from studies of the developmentally regulated miRNAs lin-4 (Lee et al., 1993; Olsen and Ambros, 1999; Wightman et al., 1993) and let-7 (Reinhart et al., 2000) in Caenorhabditis elegans. By binding and inhibiting the translation of the target mRNA, the lin-4 and let-7 RNAs play an important role in regulating the timing of larval development. Another example is bantam RNA from Drosophila melanogaster, which is expressed in a temporal and tissuespecific manner during development, suppressing apoptosis and stimulating cell proliferation by inhibiting translation of hid mRNA (Brennecke et al., 2003). In plants, miRNAs such as MIR-39 and MIR-172 show a high degree of complementarity to transcription factors that are significant in development (Aukerman and Sakai, 2003; Chen, 2003; Llave et al., 2002b; Rhoades et al., 2002). These miRNAs induce target mRNA cleavage or translational repression, thereby facilitating plant development and organogenesis.

The expression of miRNAs is often regulated in tissue-specific and developmental stage specific manners (Aravin et al., 2003; Krichevsky et al., 2003; Lagos-Quintana et al., 2002; Pasquinelli et al., 2000; Sempere et al., 2003), although the regulatory mechanism is still unknown. We have previously shown that miRNAs are transcribed as long primary transcripts (termed pri-miRNAs) (Lee et al.,

2002). These primary transcripts are first trimmed into ~70 nt stem-loop forms (called pre-miRNAs) by the RNase III type protein, Drosha, in the nucleus (Lee et al., 2003). Following this initial processing, pre-miRNAs get exported to the cytoplasm and are subject to a second processing to generate the final product of ~22 nt mature miRNAs, by another RNase III type protein Dicer. This stepwise processing and compartmentalization may allow for the fine regulation of miRNA biogenesis at multiple steps. Over three hundred miRNAs have been reported in diverse eukaryotic organisms so far (Aravin et al., 2003; Dostie et al., 2003; Grad et al., 2003; Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002; Lai et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Lee et al., 1993; Lim et al., 2003b; Llave et al., 2002a; Mourelatos et al., 2002; Park et al., 2002; Reinhart et al., 2000; Reinhart et al., 2002). The majority of miRNA genes were discovered through cDNA cloning from size-fractionated RNA samples. Recently, additional miRNA genes have been identified using computational procedures from the vertebrate, C. elegans and Drosophila. A bioinformatic study suggested that there exist 200 - 255 miRNAs in humans, accounting for almost 1% of the predicted genes (Lim et al., 2003a). If the prediction is correct, about 100 miRNA genes remain to be identified in humans because 152 miRNAs have been reported, of which 109 miRNAs have been experimentally validated (Brennecke and Cohen, 2003). miRNAs that are expressed only in specific developmental stages or conditions would be difficult to be cloned or validated.

Summary of the Invention

The present invention relates to novel microRNA molecules specifically expressed in human embryonic stem cells. The present invention provides 36 miRNAs expressed in undifferentiated hES cells, of which 17 are novel. And the present invention further provides that 14 miRNAs are expressed in an ES cell-specific manner.

Brief Description of the Figures

Fig. 1 shows predicted structures of miRNA precursors. RNA secondary structure prediction was performed using MFOLD (version 3.1) and manually refined to accommodate G/U wobble base pairs in the helical segments. The miRNA sequences are underlined.

Fig. 2 is northern blotting results of miRNAs cloned from human embryonic stem cells. Total RNA from HeLa, mouse feeder cell line (STO), mouse embryonic stem cell line (mES), two human embryonic stem cell lines (SNU-hES3 and Miz-hES1), and human embryonic carcinoma cell line (hEC) was blotted and probed with 5'-radiolabled oligodeoxynucleotide complementary to the indicated miRNA. 5S rRNA serves as a loading control. miRNAs expressed in ES cells as well as in EC cells are shadowed in yellow, while miRNAs expressed specifically in ES cells are shadowed in blue.

Fig. 3 shows multiple sequence alignment of the genomic DNA segments corresponding to the miRNA clusters. (A : hsc-1*~1~2*~2~3*~3~4~5 (chromosome

4), B : hsc-12~13~14*~14 (chromosome 19)). The positions of dominantly expressed mature miRNAs are highlighted in red and the positions of weakly expressed mature miRNAs are shown in blue. Conserved residues are indicated with asterisks. Sequences were aligned with CLUSTALW and manually refined.

Fig. 4 is RT-PCR analysis results of total RNA from HeLa, two human embryonic stem cell lines (SNU-hES3 and Miz-hES1), embryoid body derived from SNU-hES3 (SNU-hES3 (EB)) or Miz-hES1 (Miz-hES1 (EB)), differentiated cells derived from EBs (SNU-hES3 (Dif)), and human embryonic carcinoma cell line (hEC). Total RNA was reverse transcribed into firststrand cDNA, which was then subjected to PCR using primers specific to the indicated miRNAs, human Oct4, and GAPDH.

Fig. 5 is schematically depicted genomic organization of miRNA gene clusters.

Detailed Description of the Invention

We have cloned small RNAs in the range of 17-26 nt from hES cells, resulting in 36 RNAs with known characteristics of miRNAs. They were found in the stems of strong hairpin structures and the miRNA sequences as well as the secondary structures of their predicted precursors are conserved in mice. Of the 36 cloned RNAs, 16 were identical to previously reported miRNAs from various mammalian adult tissues and cell lines. The remaining 20 appeared to be novel. Recently, 15 novel miRNAs were identified from mouse ES cells (Houbaviy et al.,

2003). Comparison to this study shows that three miRNAs are common between human and mouse ES cells. Therefore, we report 17 novel miRNA genes exclusive of the 3 common miRNAs. Apart from the 3 common miRNAs, 10 novel miRNAs appear to have related sequences to the miRNAs in the mES data set (Houbaviy et al., 2003). These include hsc-1, hsc-1*, hsc-2, hsc-2*, hsc-3*, hsc-4, hsc-12, hsc-13, hsc-14, and hsc-14*. However, a considerable proportion of the cloned miRNAs from human and mouse ES cells are different from each other; 7 out of 20 miRNAs identified in our study do not have apparent homologues among those cloned from mES cells. Conversely, 5 out of 15 novel miRNAs from mES cells (Houbaviy et al., 2003) do not have related sequences in our dataset. This may be because the cloning was not intensive enough to identify the complete set of miRNAs in these cell lines. These results may also implicate fundamental differences between the regulatory networks in hES cells and mES cells.

The expression patterns of miRNAs cloned from hES cells can be classified into four groups. (1) miRNAs that are expressed in ES cells as well as in EC cells; hsc-1*, hsc-1, hsc-2*, hsc-2, hsc-3*, hsc-3, hsc-4, and hsc-5 (shadowed in yellow in Table 1 and Fig. 2). These miRNAs may have conserved roles in mammalian pluripotent stem cells. (2) miRNAs that are expressed specifically in ES cells but not in other cells including EC cells; hsc-6, hsc-7, hsc-8, hsc-12, hsc-13, hsc-14*, and hsc-14 (shadowed in blue in Table 1 and Fig. 2). These miRNAs may have functions specific to ES cells. It would be interesting to dissect the molecular basis for the differences between the two pluripotent stem cells; ES and EC cells. (3) miRNAs that are rare in ES cells but abundant in HeLa and STO cells; let-7a, miR-301 (hsc-11), hsc-16, miR-21, miR-29b, and miR-29. These miRNAs may have been

cloned from contaminated STO cells. Alternatively, they may play roles in regulation of development and differentiation, like let-7 in C. elegans (Reinhart et al., 2000). (4) The last class consists of miR-16, miR-17-5p, miR-19b, miR-26a, miR-92, miR-103, miR-130a, and miR-222. These are expressed in most tested cell lines so they may contribute to basic cellular functions (Fig. 2).

Many of the ES-specific miRNA genes are highly related to each other and organized as clusters. Clustered miRNA genes are transcribed as polycistronic primary transcripts, which are then processed through two consecutive cleavage steps by RNase III proteins, Drosha and Dicer (Lee et al., 2003). It is intriguing that two hES-specific miRNA clusters are conserved in mouse genome. Although the numbers of the homologous genes are different in human and mouse clusters, which may implicate divergence of the conserved regulatory pathways, these genes in conserved clusters are likely to play important roles in the regulation of mammalian ES cells.

Expression pattern of the cluster of miR-12~13~14*~14 (chromosome 19) is particularly interesting because down-regulation of this cluster becomes evident more rapidly than that of Oct-4, which is the earliest marker for ES cells known so far (Fig. 4A). It is tempting to speculate that these miRNAs may be the primary regulators of embryonic stem cell maintenance/differentiation, which act prior to other known factors including Oct4. These miRNAs may define the very early stage of embryonic development that has not been recognized before.

For most miRNAs, RNA from only one side of the miRNA precursor is typically cloned or detected on Northern blot. In this study, four miRNA genes (hsc-1, hsc-2, hsc-3, and hsc-14 hairpins) yielded small RNAs corresponding to both

strands of the stems (Table 1 and Fig. 1). However, the frequencies of cloning of the two opposite strands are not equal, indicating that there is a certain degree of asymmetry in the abundance of these miRNAs. In the case of hsc-1 and hsc-2, the ratios between the cloning frequencies of the two sides were 22.5:1 and 10:1, respectively. According to the standard nomenclature (Ambros et al., 2003), the less abundant miRNA of each pair was designated with an asterisk mark. Recent studies using siRNA duplexes suggested that this asymmetry may come from asymmetric degradation of the opposing strand following Dicer processing. According to this, the strand with lower internal stability at the 3' end has a better chance to survive (Khvorova et al., 2003; Schwarz et al., 2003). Because only 69% of our novel miRNA precursors were clearly in agreement with this rule, the mechanism for miRNA sequence selection during miRNA maturation may be more complex than that of siRNA strand selection.

It is intriguing that ES cells are such rich sources of novel miRNAs. Previous cloning efforts intensively searched for miRNA genes using cultured mammalian cells and adult tissues. It would be an interesting quest to look for additional miRNAs in other rare cell types. For instance, small RNA expression profiling of various stem cells or mouse embryos during development would be highly informative. ES-cell specific miRNAs appear to be less conserved than other previously reported miRNAs from adult tissues and cell lines. miRNAs found in our study have their homologues only in other mammals but not in invertebrates such as C. elegans and Drosophila. The low degree of conservation may partially explain the reason why these miRNAs have not been identified until now. They may have escaped bioinformatics database searches, which partially rely on

phylogenetic conservation.

None of the miRNA cloned in our study complements perfectly to known mRNAs or ESTs, suggesting that these miRNAs may act as translational inhibitors through imperfect pairing to their target mRNAs. It would be of great importance to identify the target mRNAs of each novel miRNA, which will lead us to understand the complex and interesting networks of regulation in ES cells.

<Materials and methods>

Human ES cell culture

Human ES cells (SNU-hES3 and MIZ-hES1) were maintained in DMEM/F12 (Gibco BRL) supplemented with 20 % (v/v) serum replacements (Gibco BRL), penicillin (100 i.u/ml,Gibco BRL) and streptomycin (100 μg/ml, Gibco BRL), 0.1 mM nonessential amino acids(Gibco BRL), 0.1 mM Mercaptoethanol (Sigma) and 4 ng/ml basic FGF (R&D). Media was changed daily. Human ES cell colonies were cultured on a feeder layer of mouse STO (ATCC CRL-1503) cells pre-treated with mytomycin C (Sigma) and were manually detached and transferred onto new STO feeders every 5-6 days. HS-3 mouse ES cells were grown under standard condition.

Differentiation of human ES cells

To prepare embryoid bodies (EBs), the whole colonies of hES cells were detached by glass pipette, transferred onto petri dishes coated with pluronic F-127 (Sigma), and incubated for 10 days. The media for EB was identical to the hES

media except that it lacked bFGF. Every two days, the media was changed by using a pipette. To further differentiate EBs made from SNU-hES3, they were plated onto tissue culture plates coated with poly-L-ornithin (0.01% (v/v))/fibronectin (5 g/ml (w/v)). Cells were further incubated for 5 days in N2 supplement medium containing 20 ng/ml bFGF and the medium was changed daily. Confluent cells were manually detached, and then pipetted using yellow tips and transferred onto new plates coated with poly-L-ornithin/fibronectin. Cells were cultured for 5 days in N2 medium containing 20 ng/ml bFGF. When the cells reached confluency, they were trypsinized and split 2:1 or 3:1 at new poly-L-ornithin/fibronectin-cotated plates.

miRNA cloning and bioinformatic analysis.

Total RNA was prepared from each cell lines with TRIzol regent (Gibco-BRL).

Cloning of miRNA was performed as described (Lagos-Quintana et al., 2001). Database searches were performed at the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) and ENSEMBL server (http://www.ensembl.org). Genomic sequences covering the region identical to the cloned miRNAs were examined by using MFOLD (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) to predict the secondary structure. Sequence alignment between miRNA sequences were performed by using CLUSTALW (http://www.ebi.ac.uk/clustalw/).

Northern blot analysis.

Total RNA (100 μg) from each cell line was loaded on a 12.5% denaturing

polyacrylamide gel. The resolved RNA was transferred to a Zeta-Probe GT blotting membrane (Bio-Rad) overnight. Oligodeoxinucleotides labeled at the 5' end with 32P-γ-ATP were used as probes. Prehybridization and hybridization was carried out using Express Hyb Hybridization Solution (Clontech) according to the manufacturer's instruction. The sequence of the probes is shown in Table 1.

Table 1. The sequences of the probes used in Northern blot analysis

	Sequence (5' → 3')	SEQ ID NO.
hsc-1*	AGAAAGCACTTCCATGTTAAAGT	1
hsc-1	CTACTAAAACATGGAAGCACTTA	2
hsc-2*	CAGCAGGTACCCCCATGTTAAA	3
hsc-2	CCACTGAAACATGGAAGCACTTA	4
hsc-3*	AAAGCAAGTACTACCACGTTTA	5
hsc-3	TCACCAAAACATGGAAGCACTTA	6
hsc-4	ACACTCAAACATGGAAGCACTTA	7
hsc-5	TCACCATTGCTAAAGTGCAATT	8
hsc-6	TCCATCATTACCCGGCAGTATTA	9
hsc-7	AAACGTGGAATTTCCTCTATGT	10
hsc-8	AATAGGTCAACCGTGTATGATT	11
hsc-9	AAAGATCAACCATGTATTATT	12
hsc-10	CCAGGTTCCACCCCAGCAGGC	13
hsc-11	GCTTTGACAATACTATTGCACTG	14
hsc-12	ACACTCAAAAGATGGCGGCAC	15
hsc-13	ACGCTCAAATGTCGCAGCACTTT	16
hsc-14	ACACCCCAAAATCGAAGCACTTC	17
hsc-14*	GGAAAGCGCCCCCATTTTGAGT	18
hsc-15	ACAGGATTGAGGGGGGGCCCT	19
hsc-16-	CACTTATCAGGTTGTATTATAA	20
Let-7a	AACTATACAACCTACTACCTCA	21
miR-16	CGCCAATATTTACGTGCTGCTA	22
miR-17-5p	ACTACCTGCACTGTAAGCACTTTG	23

miR-19b	TCAGTTTTGCATGGATTTGCACA	24
miR-21	GTCAACATCAGTCTGATAAGCTA	25
miR-26a	AGCCTATCCTGGATTACTTGAA	26
miR-29	AACCGATTTCAGATGGAGCTAG	27
miR-29b	CACTGATTTCAAATGGTGCTA	28
miR-92	CAGGCCGGGACAAGTGCAATA	29
miR-103	CATAGCCCTGTACAATGCTGCT	30
miR-124a	TGGCATTCACCGCGTGCCTTAA	31
miR-130a	ATGCCCTTTTAACATTGCACTG	32
miR-134	CCCCTCTGGTCAACCAGTCACA	33
miR-135-2	TCACATAGGAATAAAAAGCCATA	34
miR-136	TCCATCATCAAAACAAATGGAGT	35
miR-222	GAGACCCAGTAGCCAGATGTAGCT	36

RT-PCR

Two to five micrograms of total RNA from the indicated cells was used for the firststrand cDNA synthesis with SUPERSCRIPT II (Gibco-BRL). The primer pairs specific to hsc-1*~1~2*~2~3*~3~4~5, hsc-12~13~14*~14, miR-30a, let-7a-1, Oct4, GAPDH are as shown in Table 2 below.

Table 2. The primer used in RT-PCR

	Primer	Sequence(5' → 3')	SEQ ID NO.
hsc-1*~1~2*~2~3*~3~4~5	Forward	GGGCTCCCTTCAACTTTAAC	37
	Reverse	ATTCTGTCATTGGCTTAACAATCCATCACC	38
hsc-12~13~14*~14	Forward	CGATCGCCGCCTTGCCGCAT	39
	Reverse	TGGTTCGTGATGCCCTACTCAAACAGGGAC	40
miR-30a	Forward	ATTGCTGTTTGAATGAGGCTTCAGTACTTT	41
	Reverse	TTCAGCTTTGTAAAAATGTATC AAGAGAT	42
let-7a-1	Forward	GATTCCTTTTCACCATTCACCCTGGATGTT	43
	Reverse	TTTCTATCAGACCGCCTGGATGCAGACTTT	44
Oct4	Forward	CTGAAGCAGAAGAGGATCAC	45

	Reverse	GACCACATCCTTCTCGAGCC	46
GAPDH	Forward	TGTCATCAATGGAAATCCCATCACC	47
	Reverse	CATGAGTCCTTCCACGATACCAAA G	48

<Results>

MicroRNAs cloned from human embryonic stem cells

To identify miRNAs expressed in hES cells, two independent cDNA libraries were generated by directional cloning method using size fractionated RNA (17-26 nt) from undifferentiated hES cells, SNU-hES3 (registered at the Korea Stem Cell Research Center). hES cells were grown on a feeder layer of the STO cell line pre-treated with mitomycin C. To assess the undifferentiating status, the steady-state level of Oct4 mRNA was determined by RT-PCR, and the cells were stained for alkaline phosphatase. In addition, the expression of cell surface markers including stage-specific embryonic antigen SSEA-1, SSEA-3, and SSEA-4 was checked. The undifferentiated SNU-hES3 cells expressed a high level of Oct4 (Fig. 4),alkaline phosphatase, SSEA-3, and SSEA-4 throughout this study (data not shown).

Sequences were obtained for 1475 small cDNAs that resulted in 733 non-redundant sequences. Approximately 70% of these sequences corresponded to the expressed strand of loci that encode longer, previously identified coding or noncoding RNAs such as tRNAs and rRNAs. To distinguish miRNAs from degradation products or small interfering RNAs (siRNAs), we evaluated the ability of RNA containing the clones to fold into stem-loop by using the MFOLD program. Thirty-six RNAs were found in the stems of strong hairpin structures (Table 1 and Fig. 1). Although some abundant RNAs appeared as many as 45 times,half of the

potential miRNAs were represented by only one clone, suggesting that these RNAs are relatively less abundant in hES cells and/or that our miRNA dataset may not represent the complete pool of miRNAs present in hES cells. There was no siRNA-like clone as none of the clones appeared to be originated from long dsRNA or repetitive sequences. Of the 36 cloned RNAs, 16 were identical to previously reported miRNAs from various mammalian adult tissues and cell lines (Table 1, lower panel). Interestingly, the remaining 20 RNAs were represented by the majority (77%) of the clones (122 of 158 clones) (Table 1,upper panel). This presents a striking contrast to a recent study using a similar cloning method, where 91% of ~600 clones matched previously identified miRNAs (Lagos-Quintana et al.,2003). The primary difference was in the source of RNA; various tissues of 18.5-week-old adult mice and the human osteoblast sarcoma cell line Saos-2 (Lagos-Quintana et al., 2003).

In accordance with the prediction that some cells such as the cells in early development may express specific miRNAs (Lagos-Quintana et al., 2003), our data show that hES cells are highly enriched with a distinct set of miRNAs unlike the cells in adult tissues or other cell lines.

Recently, 15 novel miRNAs were reported from mouse ES cells (Houbaviy et al., 2003). Comparison to this study shows that 3 miRNAs are common between the novel miRNA data sets from mouse and human ES cells; miR-296 (hsc-15), miR-301 (hsc-11) and miR-302 (hsc-3). Thus, we here report 17 novel miRNA genes exclusive of the 3 common genes. MicroRNAs cloned from human embryonic stem cells are shown in Table 3 below.

Table 3. MicroRNAs cloned from human embryonic stem cells.

ID[a]	Sequence	Observation[b] 1st	2nd	Size	Chromosome	Conservation[c]	Expression[d]	SEQ ID NO.
hsc-1*	ACUUUAACAUGGAAGUGCUUUCU	1	1	23	4	Mm	00011	49
hsc-1	UAAGUGCUUCCAUGUUUUAGUAG	9	36	23	4	Mm	00011, 000221	50
hsc-2*	UUUAACAUGGGGGUACCUGCUG	1		22	4	Mm	00011	51
hsc-2	UAAGUGCUUCCAUGUUUCAGUGG	7	3	23	4	Mm	00012	52
hsc-3*	UAAACGUGGAUGUACUUGCUUU	3	3	22	4	Mm	00012	53
hsc-3 (miR-302)	UAAGUGCUUCCAUGUUUUGGUGA	6	17	23	4	Mm, Rn	00023, 001223	54
hsc-4	UAAGUGCUUCCAUGUUUGAGUGU	1	12	23	4	Mm	00023, 000112	55
hsc-5	AAUUGCACUUUAGCAAUGGUGA	2	3	22	4	Mm, Rn	00011	56
hsc-6	UAAUACUGCCUGGUAAUGAUGA	2	4	23	12	Mm	00010, 000110	57
hsc-7	ACAUAUAGGAAAUCCCACGUUC	1		22	14	Mm	000105, 000110	58
hsc-8	AAUCAUACAGGUUGAUCUAUUU	1	1	22	14	Mm	000110	59
hsc-9	AAUAAUACAUGGUUGAUCUUU	1		21	14	Mm	no signal	60
hsc-10	GCCUGCUGGGGUGGAACCUGG		1	21	14	Mm	00S000	61
hsc-11 (miR-301)	CAGUGCAAUAGUAUUGUCAAAGC	1		23	17	Mm Rn, Fr	21111	62
hsc-12	GUGCGCCAUCUUUUGAGUGU		2	21		Mm	002120	63
hsc-13	AAACUGCCUGCGAGAUUUGAGCGU		1	23	9	Mm	000230	64
hsc-14	AGUCAAAAUGGGGCGCUUUCC		1	22	9	Mm	00SSS0	65
hsc-14	GAAGUGCUUCGAUUUUGGGUGU		1	23	19	Mm	000120	66
hsc-15 (miR-296)	AGGGCCCCCCCUCAAUCCUGU		1	21	20	Mm	00SSS	67
hsc-16	UUAUAAUACAACCUGAUAAGUG	2		22	X	Mm	11000	68
sum		35	87					
let-7a	UGAGGUAGUAGGUUGUAUAGUU		1	22	9, 11, 17, 22		33000	69
miR-16	UAGCAGCACGUAAAUAUUGGCG		2	22	13		22112	70
miR-17-5p	CAAAGUGCUUACAGUGCAGGUAGU		1	24	13		11112	71
miR-19b	UGUGCAAAUCCAUGCAAAACUGA	7	1	23	13, X		111111	72

miR-21	UAGCUUAUCAGACUGAUGUUGAC		4	23	17		32111	73
miR-26a	UUCAAGUAAUCCAGGAUAGGCU		3	22	3		221112	74
miR-29	CUAGCACCAUCUGAAAUCGGUU		1	22	7		12SSSS	75
miR- 29b-2	UAGCACCAUUUGAAAUCAGUG		1	21	7		11000	76
miR-92	UAUUGCACUUGUCCCGGCCUG	1	1	21	13, X		11112	77
miR-103	AGCAGCAUUGUACAGGGCUAUG	1	2	22	5		21112	78
miR-124a #	UUAAGGCACGCGGUGAAUGCCA	3	1	22	8		00SSS	79
miR-130a #	CAGUGCAAUGUUAAAAGGGCAU		1	22	11		12223	80
miR-134 #	UGUGACUGGUUGACCAGAGGGG	1		22	14		00SSS0	81
miR-135-2 #	UAUGGCUUUUUAUUCCUAUGUGA		1	23	12		no signal	82
miR-136 #	ACUCCAUUUGUUUUGAUGAUGGA	1		23	14		no signal	83
miR-222	AGCUACAUCUGGCUACUGGGUCUC	1	1	24	X		11SSS1	84
sum		15	21					

[a] : miRNAs that were newly identified in this study are listed in the upper panel. The exceptions are the miRNAs recently cloned from mES cells and indicated in the brackets. Lines shadowed (hsc-6, hsc-7, hsc-8) indicate the miRNAs expressed in both ES cells and EC cells. Lines shadowed (hsc-12, hsc-13, hsc-14*, hsc-14) indicate the miRNAs expressed in ES cells but not in other cells including EC cells.

[b] : The longest clone is presented.

[c] : Number of the clones found in each library.

[d] : The presence of homologous stem loops in the mouse (Mm), rat (Rn), and pufferfish (Fr) is indicated. Note that these homologues are only predicted ones based on the genomic sequences.

[e] : Expression patterns determined by Northern blot analysis. Single digit numbers indicate the relative band intensities of given miRNA in different cell lines as shown

in Figure 2 and do not give information about the relative levels of different miRNAs. Five digits represent band intensities from HeLa, STO, mES, SNU-hES1, and hEC, consecutively. Six digits represent band intensities from HeLa, STO, mES, SNU-hES1, Miz-hES3, and hEC, consecutively. S indicates a smear around ~22nt that makes it difficult to judge the expression level of the given miRNA.

Most of the novel miRNAs from hES are expressed specifically in hES cells

To validate the expression of the cloned miRNAs, Northern blot analysis was performed using total RNAs from undifferentiated hES cells, mES cells and the hEC cell line, NTERA2. To confirm the expression of miRNAs from hES cells, we employed another hES cell lie, MIZ-hES1 that is registered in the NIH Human Embryonic Stem Cell Registry. As negative controls, total RNAs from HeLa cells and STO feeder cells were used (Fig. 2). Of the 17 newly identified miRNAs, 14 miRNAs are expressed in a hES cell-specific manner; hsc-1*, hsc-1, hsc-2*, hsc-2, hsc-3*, hsc-4, hsc-5, hsc-6, hsc-7, hsc-8, hsc-12, hsc-13, hsc-14*, and hsc-14. miR-302 (hsc-3) that was previously reported in mES cells is also expressed specifically in mES, hES, and hEC cells. miR-296 (hsc-15) appeared as smear which made it difficult to judge the specificity of expression. Two miRNAs showed no ES cell-specific expression; miR-301 (hsc-11) was detectable in all samples tested and hsc-16 was mainly expressed in HeLa and STO cell lines. The remaining two clones (hsc-9 and hsc-10) could not be detected. They might be expressed only at very low levels.

Polycistronic nature of hES cell specific-miRNA genes

Twelve miRNA genes were found to be part of two gene clusters. Eight miRNAs (hsc-1, hsc-1*, hsc-2, hsc-2*, hsc-3, hsc-3*, hsc-4, and hsc-5) are located within an about 700 bp region on chromosome 4. Another four miRNAs (hsc-12, hsc-13, hsc-14, and hsc-14*) are found within a 1050 bp region on chromosome 19. Sequence comparison of these miRNAs shows that the miRNAs in a given cluster are related (Fig. 3). Four miRNAs of chromosome 4 cluster (hsc-1, hsc-2, hsc-3 (miR-302), and hsc-4) are highly homologous to each other. Their sequence similarity is greatest in the 5' portions of the miRNA sequences as is the case with the lin-4 and let-7 families. This finding is consistent with the hypothesis that target recognition occurs primarily via 5' sequences (Lai, 2002). These related miRNAs may recognize a consensus target sequence and hence act on the same mRNAs or different mRNAs with conserved binding sites. Therefore, recognition of these miRNA gene families should help in the identification of putative mRNA targets. The consensus sequence for these clustered miRNAs is 5'-UAAGUGCUUCCAUGUUUNNGUNN-3' (Fig. 3A). While these miRNAs are most abundant ones in hES cells, their murine homologue miR-302 appears to be less abundant in mES cells (Houbaviy et al., 2003). It is noted that miR-302 and additional sequences which are related to this family are found in mouse chromosome 3 as a cluster although they have not been identified experimentally (Table 1). Three other related miRNAs (hsc-12, hsc-13, and hsc-14) are also found in a cluster on chromosome 19 (Fig. 3B). Their mouse homologues (miR-291~295) also have related sequences and are located as one cluster spanning 2.2 kb (Houbaviy et al., 2003). These conserved miRNA gene families are likely to play central roles

in mammalian ES cells.

To examine the expression patterns of the miRNA clusters during hES cell differentiation, RT-PCR was carried out. Because clustered miRNA genes are generally transcribed into polycistronic primary transcripts (pri-miRNAs) (Lee et al., 2002), the primers were chosen to bind outside the boundary of the predicted ~70 nt stem-loop clusters so that we could detectri-miRNAs covering the entire cluster. PCR products of the expected size (707 and 1056 bp, respectively) were detected from two hES cell lines (Fig. 4A, lanes 2 and 5), indicating that these clusters are indeed single transcriptional units.

Clustered miRNAs are rapidly down-regulated during differentiation

The steady-state levels of primary transcripts from the two gene clusters decreased in human embryoid bodies (EBs) (Fig. 4A, lanes 3 and 6). EBs were prepared by culturing hES cells for 10 days in the presence of hES cultured media without bFGF. The levels were reduced further in differentiated cells derived from EBs (Fig. 4A, lane 4). These results demonstrate that the clustered miRNAs are expressed specifically in hES cells and are rapidly down-regulated during differentiation. To assess the degree of differentiation, we determined the steady-state levels of Oct4 mRNAs by RT-PCR (Fig. 4A). Interestingly, down-regulation of chromosome 19 cluster (miR-12~13~14*~14) precedes that of Oct4. The polycistronic transcripts from the chromosome 4 cluster, but not those from the chromosome 19, were detected in hEC cells (Fig. 4A, lane 7), which is consistent with the results from Northern blot analysis (Fig. 2). The control miRNA (miR-

30a), which had not been cloned from hES cells, was detected in HeLa cells but only barely shown in other cells in accordance with Northern results (Fig. 2 and 4). Pri-let-7a-1 transcript was expressed in all of the tested cell lines (Fig. 4) although mature let-7a-1 appeared only in HeLa and STO cell lines (Fig. 2), which suggests that the processing of let-7a-1 may be regulated posttranscriptionally. It would be of great interest to understand how the expression of miRNAs is regulated along the developmental stages.

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FIG. 1

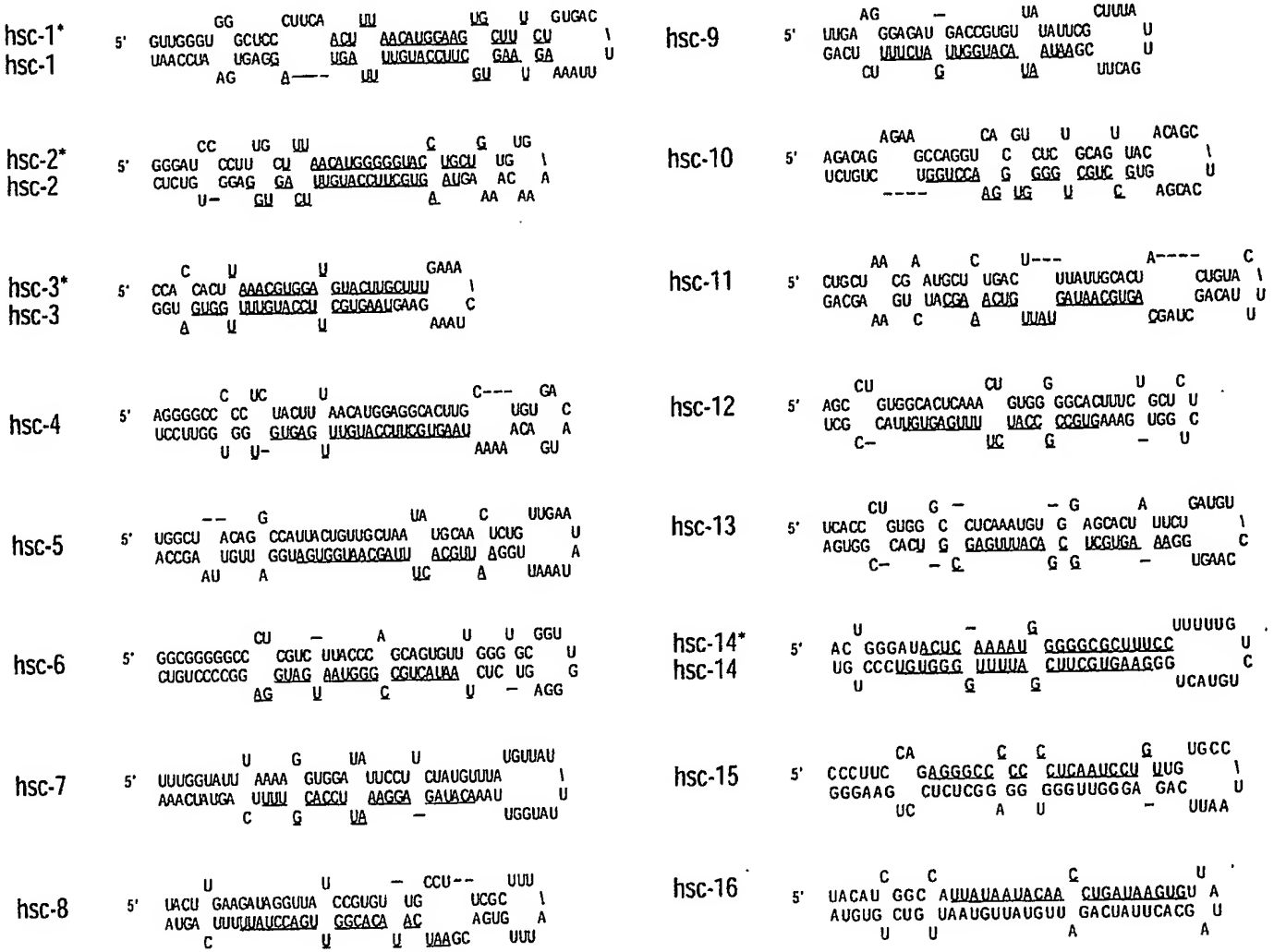


FIG. 2

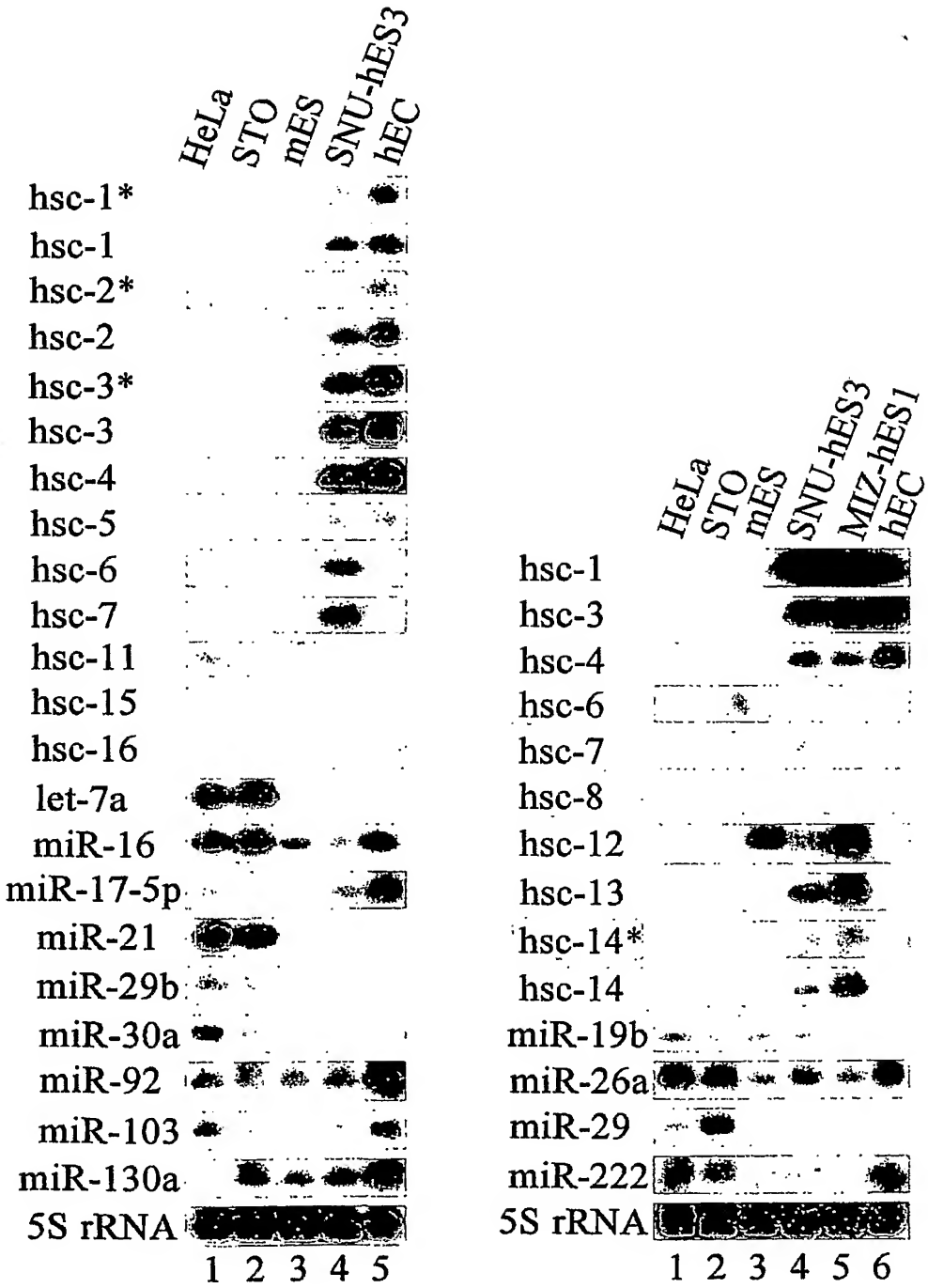


FIG. 3

A hsc-1*~1~2*~2~3*~3~4~5 (chromosome 4)


```
hsc-1* and 1    CAACTTTAACATGGAAGTGCTTTCTGTG--ACTTTAAAAGTAAGTGCTTCCATGTTTTAGTAGGA-
hsc-2* and 2    --GCTTTAACATGGGGGTACCTGCTGTG-TGAAACAAAAGTAAGTGCTTCCATGTTTCAGTGGAGG
hsc-3* and 3    ---CTTAAACGTGGATGTACTTGCTTTGAAACTAAAGAAGTAAGTGCTTCCATGTTTTGGTGATGG
hsc-4           --ACTTTAACATGGAGGCACTTGCTGTGACATGACAAAAATAAGTGCTTCCATGTTTGAGTGTGG-
                   *** *** ***  *  * * ** **       * ** ******************  **
concensus                                                  TAAGTGCTTCCATGTTTNNGTNN
```

B hsc-12~13~14*~14 (chromosome 19)


```
hsc-12          TGTGGCACTCAAACTGTGGGGGCACTTTCTGCTCTCTG-GT--GAAAGTGCCGCCATCTTTTGAGTGTTA
hsc-13          TGTGGGCCTCAAA-TGTGGAGCACTATTCTGATGTCCA-AGTGGAAAGTGCTGCGA-CATTTGAGCGTCA
hsc-14* and 14  -GGGATACTCAAAATGGGGGCGCTTTCCTTTTTGTCTGTACTGGGAAGTGCTTCGA-TTTTGGGGTGTCC
                 * *   *** *** ** **          *  * **       * *** ***  * *   ** * * **
concensus                                                   GTGCNNCNA-NNTTNGNGNGT
```

FIG. 4

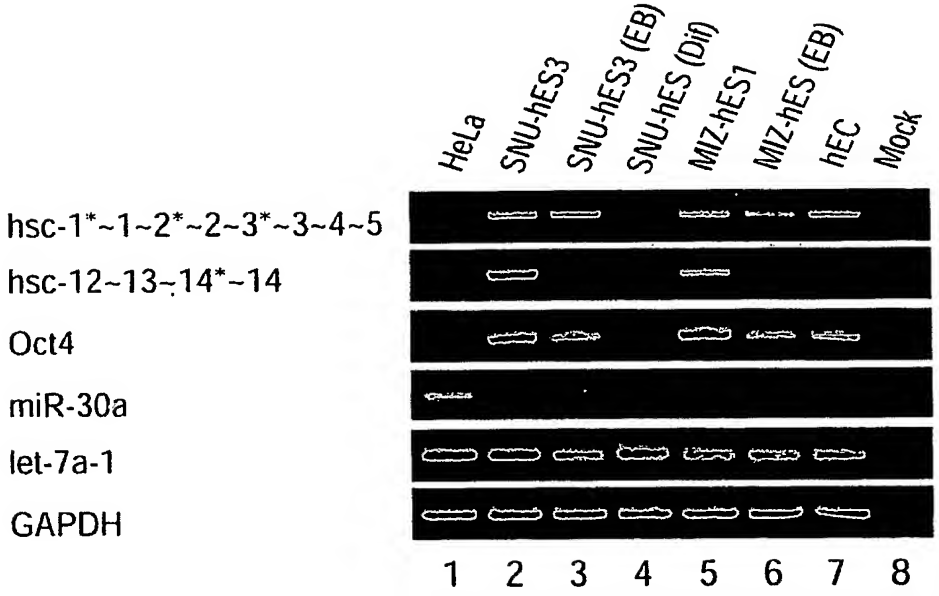
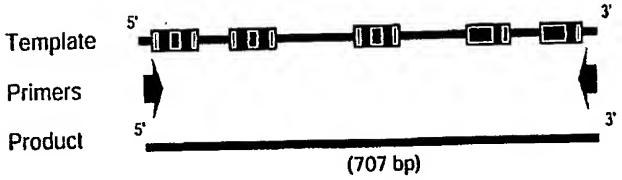
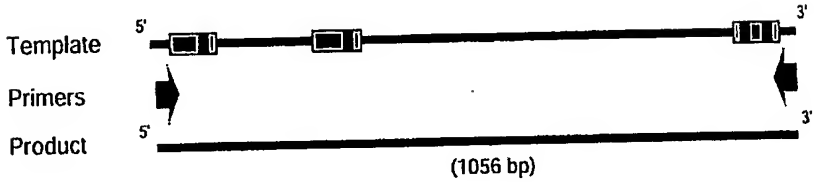


FIG. 5

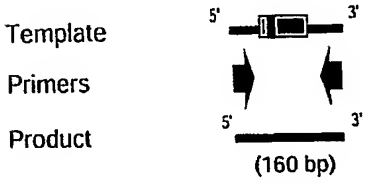
a) hsc-1*~1~2*~2~3*~3~4~5 (chromosome 4)



b) hsc-12~13~14*~14 (chromosome 19)



c) let-7a-1



d) miR-30a~30a*